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Neurogenin2 Specifies the Connectivity of Thalamic Neurons by Controlling Axon Responsiveness to Intermediate Target Cues

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Summary

Many lines of evidence indicate that important traits of neuronal phenotype, such as cell body position and neurotransmitter expression, are specified through complex interactions between extrinsic and intrinsic genetic determinants. However, the molecular mechanisms specifying neuronal connectivity are less well understood at the transcriptional level. Here we demonstrate that the bHLH transcription factor *Neurogenin2* cell autonomously specifies the projection of thalamic neurons to frontal cortical areas. Unexpectedly, *Ngn2* determines the projection of thalamic neurons to specific cortical domains by specifying the responsiveness of their axons to cues encountered in an intermediate target, the ventral telencephalon. Our results thus demonstrate that in parallel to their well-documented *proneural* function, bHLH transcription factors also contribute to the specification of neuronal connectivity in the mammalian brain.

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Introduction

Significant progress has been made in identifying the extracellular cues, corresponding receptors, and intracellular signaling pathways involved in guiding axons to their final target in the developing nervous system (Dickson, 2002; Flanagan and Vanderhaeghen, 1998; Kolodkin, 1998; Tessier-Lavigne and Goodman, 1996). Paradoxically, however, the molecular mechanisms specifying neuronal connectivity are poorly understood at the transcriptional level. In contrast, a number of intrinsic programs involved in specifying other aspects of neuronal identity have been identified and shown to depend upon the combinatorial expression of different classes of transcription factors, including many homeodomain proteins (Jessell, 2000; Kania et al., 2000; Sharma et al., 2000; Shirasaki and Pfaff, 2002; Thaler et al., 2002). Recent evidence suggests that bHLH transcription factors also participate in neuronal subtype specification in addition to their well-documented *proneural* function (Bertrand et al., 2002). In *Drosophila,* the proneural gene *atonal*, which is closely related to the mammalian *Neurogenins (Ngns),* has been shown to regulate axon arborization and pathfinding through a *Notch*-dependent signaling pathway (Giniger, 1998; Giniger et al., 1993; Hassan et al., 2000). Recently, *atonal* has been shown to specify axon responsiveness by regulating *Robo3* expression (Zlatic et al., 2003). In mammals, close homologs of *atonal* such as *Ngn1* and *Ngn2* display a clear *proneural* activity and have also been implicated in neuronal subtype specification (Fode et al., 2000; Lo et al., 2002; Ma et al., 1999; Perez et al., 1999; Zirlinger et al., 2002). Interestingly, in the developing spinal cord, as well as in other regions of the nervous system, bHLH transcription factors have been shown to control multiple aspects of neurogenesis, including the specification of neuronal versus glial fate, neuronal phenotype, and the timing of cell cycle exit (Bertrand et al., 2002). There is also evidence that bHLH transcription factors act in a cooperative manner, with *Ngn2* and *Olig2*, for example, acting together to specify specific motorneuron subtypes (Mizuguchi et al., 2001; Novitch et al., 2001; Scardigli et al., 2001; Zhou and Anderson, 2002). Taken together, these results suggest that bHLH transcription factors such as *Ngn2* may coordinate the acquisition of generic neuronal features (*proneural* effect) and the specification of a particular neuronal subtype identity (Novitch et al., 2001).

To assess the potential role of *Ngn2* in the specification of neuronal connectivity, we chose to examine the thalamocortical projection system for two reasons: (1) recent reports had revealed a strikingly regionalized pattern of *Ngn2* expression in the developing dorsal thalamus, suggesting a role in thalamic neuronal subtype specification (Nakagawa and O'Leary, 2001), and (2) the molecular mechanisms that orchestrate the exquisitely precise topography of thalamic neuron projection to unique sets of cortical areas remain poorly understood.

Each thalamic nucleus is composed of a subset of neurons projecting to a unique set of cortical areas. The topography of thalamic projections is organized along the rostro-caudal and the lateromedial axis (Caviness and Frost, 1980; Crandall and Caviness, 1984; Hohl-Abrahao and Creutzfeldt, 1991). First, axons originating from *rostral* thalamic nuclei project to *rostro-medial* cortical areas, whereas *caudal* thalamic nuclei projects *caudo-laterally* in the cortex. Second, axons originating from lateral thalamic nuclei project more *caudally* in the cortex than axons originating from medial nuclei. This coordinate transformation leads to the basic patterning of thalamocortical connections linking each thalamic nucleus with a unique set of cortical areas found in rodents, carnivores, and primates (Hohl-Abrahao and Creutzfeldt, 1991).

How is this complex topography of axonal projections patterned during development? The answer to this question remains unclear. The reciprocal connectivity of a given thalamic nucleus with a unique set of cortical areas, as well as the close physical proximity of corticothalamic (CT) and thalamocortical (TC) axons in the internal capsule (IC) of developing rodents, led to the proposal of the "handshake" model whereby CT and TC axons interact in the IC to control each other's guidance (Blakemore and Molnar, 1990; McConnell et al., 1989; Molnar et al., 1998a; Molnar and Blakemore, 1995a). However, few experimental results support this model, with the noticeable exception of several lines of mutant mice (such as *Gbx-2* or *Tbr-1*) displaying defects in either TC or CT pathfinding and consequently affecting the pathfinding of the reciprocal populations of CT or TC axons (Hevner et al., 2002). Additional transcription factors have also been implicated in the control of TC pathfinding, including *Mash1, Ebf1*, *Dlx1/2*, *Pax6*, and *Emx2* (Garel et al., 1999, 2002; Hevner et al., 2002; Jones et al., 2002; Lopez-Bendito et al., 2002; Pratt et al., 2000; Tuttle et al., 1999). However, the exact role of these genes in patterning TC projections is often difficult to interpret because of their complex spatiotemporal expression patterns. In fact, most of the above-mentioned transcription factors are not only expressed in the thalamus and/or the cortex but also along the pathway of TC axons (see for example, Garel et al., 2002). Therefore, in the absence of a reliable assay recapitulating the topography of thalamocortical connections in vitro, it has been difficult to determine if these genes exert their function cell autonomously or nonautonomously during development.

In the present study, we demonstrate that the bHLH transcription factor *Ngn2* cell autonomously specifies the projection of thalamic axons to frontal cortical areas by controlling axon responsiveness to cues encountered at the level of an intermediate target, the *ventral telencephalon*. Our results demonstrate that (1) in parallel to their well-documented *proneural* function, bHLH transcription factors also specify neuronal connectivity during development of the mammalian central nervous system and (2) that the topography of thalamocortical projections to specific cortical domains is initiated by topographic cues located in an intermediate target.

Results

Neurogenin2 Is Expressed Regionally in the Developing Dorsal Thalamus

The bHLH transcription factor *Ngn2* had previously been reported to be expressed in the dorsal thalamus (DT) in a high-rostral to low-caudal gradient (Fode et al., 2000; Nakagawa and O'Leary, 2001; Sommer et al., 1996). We extended these studies by performing a detailed spatial and temporal analysis of *Ngn2* expression using in situ hybridization (ISH). At embryonic day (E) 13.5, the DT primarily consists of a proliferating neuroepithelium, with most progenitors undergoing terminal divisions (Angevine, 1970). Figure 1A shows that in the horizontal plane of section, *Ngn2* transcripts were detected in progenitors in the rostral-most third of the dorsal thalamus as early as E13.5. At E14.5 (Figure 1B), *Ngn2* expression was initiated in postmitotic neurons that are restricted to the rostral tip of the DT mantle (star in Figure 1B), whereas the remaining neuroepithelium showed a pronounced decrease of expression as compared to E13.5 (arrow in Figure 1B). Finally at E15.5 (Figure 1C), when neurogenesis is complete, *Ngn2* was still expressed in the rostral third of the DT (star in Figure 1C) but also extended caudally to the medial part of the DT. The highly regionalized expression of *Ngn2* in postmitotic neurons at E15.5 is surprising given that *Ngn2* is best characterized for its proneural activity exerted at the level of dividing neural precursors (reviewed in Bertrand et al., 2002). These results suggest that *Ngn2* may play a late role in neuronal differentiation.

In order to get more insight into the role of *Ngn2* in neuronal differentiation, we genetically knocked in an IRES-EGFP construct into the endogenous open reading frame of *Ngn2* by homologous recombination in ES cells ($Ngn2^{KIGFP}$allele ; C.S., G.G., and F.G., unpublished data). We first wanted to determine if the pattern of expression of GFP in $Ngn2^{KIGFP/+}$ transgenic mice recapitulated the spatial and temporal pattern of expression of the endogenous gene. In E15.5 $Ngn2^{KIGFP/+}$ embryos, the GFP transgene was expressed in the DT in a high-rostral to low-caudal gradient (Figures 1D–1F), recapitulating the gradient of expression visualized using ISH at E14.5 (Figure 1C). The high-rostral to low-caudal expression pattern was maintained at E16.5 (Figures 1G–1G″). Interestingly, GFP was also enriched in axons traveling through the IC at E16.5 (Figures 1H′–1I′). GFP was specifically enriched in axons projecting rostrally (Figures 1H–1H″) and dorsally (Figures 1I–1I″) in the IC at the level of the ventral telencephalon. At E17.5, the graded expression of GFP in the DT is still visible (circled in Figure 2A), but note that the transgene is not expressed in a detectable gradient but rather uniformly along the rostro-caudal axis in the cortex (Figure 2A).

Ngn2 Is Expressed by Thalamic Neurons Projecting to Rostral Cortical Areas

To examine the fate of progenitors and neurons expressing *Ngn2* in the rostral territory of the DT (Figure 2A) during embryonic development, we used GFP as a short-term lineage tracer. To do this, we determined which thalamic nuclei expressed GFP in $Ngn2^{KIGFP/+}$ mice

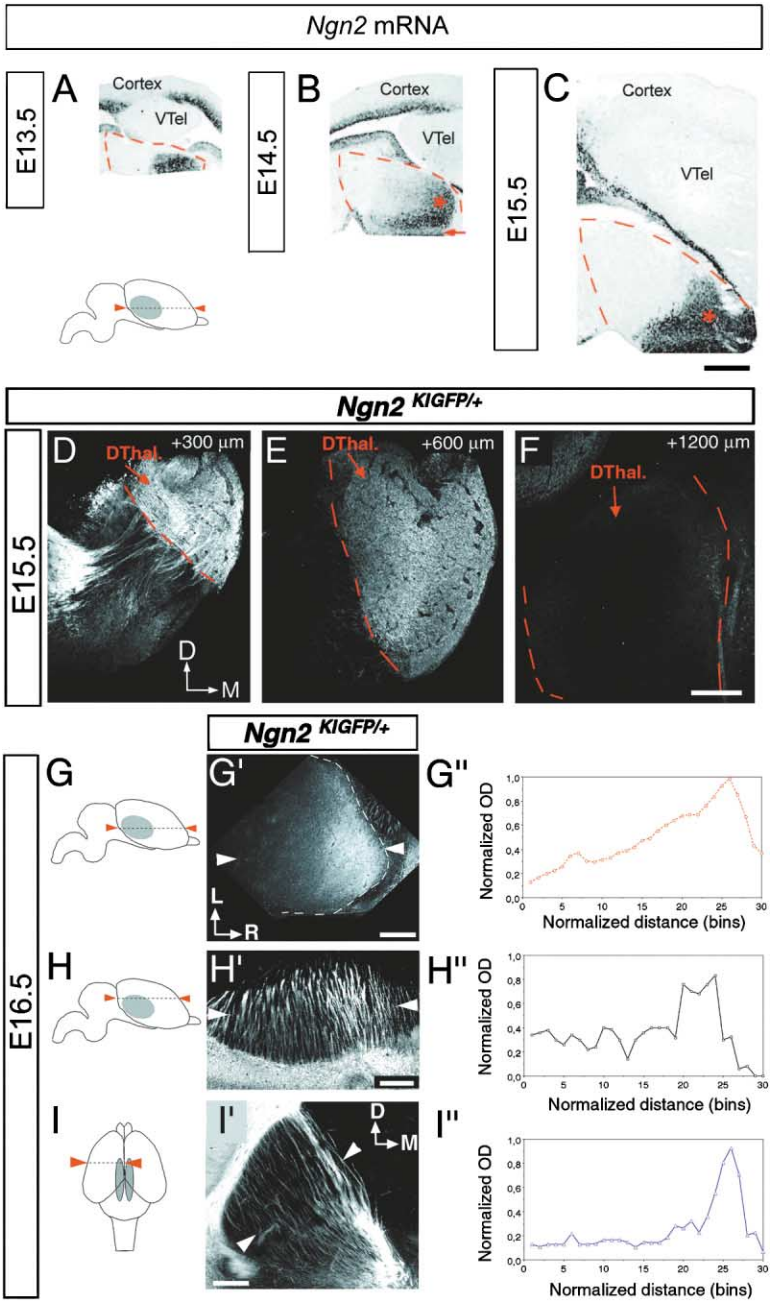

Figure 1. Spatially and Temporally Regionalized Pattern of Expression of *Neurogenin2* in the Dorsal Thalamus

(A–C) In situ hybridization performed on horizontal sections demonstrates the pattern of expression of *Neurogenin2* mRNA in the rostral tip of the developing dorsal thalamus at E13.5 (A), E14.5 (B), and E15.5 (C).

(D–F) Coronal sections of a *Ngn2*$^{KIGFP/+}$ E15.5 embryo illustrating the graded expression of GFP from high-rostral (D) to intermediate-medial (E) to low-caudal (F) levels in the dorsal thalamus. The level of section relative to the first section passing through the dorsal thalamus is indicated in (D), (E), and (F).

(G–I) Schemas illustrating the plan of sections used in (G′) to (I′), respectively (horizontal for G′ and H′; coronal for I′).

(G′ and G″) Qualitative aspect (G′) and quantitative measure (G″) of normalized optical density demonstrating the high-rostral to low-caudal gradient of expression of GFP in the dorsal thalamus in E16.5 *Ngn2*$^{KIGFP/+}$ embryos.

(H′ and H″) Qualitative aspect (H′) and quantitative measure (H″) of normalized optical density demonstrating the high-rostral to low-caudal gradient of expression of GFP in axons of the internal capsule in a E16.5 *Ngn2*$^{KIGFP/+}$ embryo.

(I′ and I″) Qualitative aspect (I′) and quantitative measure (I″) of normalized optical density demonstrating the high-dorsal to low-ventral gradient of expression of GFP among axons of the internal capsule of a E16.5 *Ngn2*$^{KIGFP/+}$ embryo.

Scale bars equal 300 μm (A–C), 150 μm (D–F), 500 μm (G′ and H′), and 400 μm (I′).

at postnatal day 1 (P1) when thalamic nuclei are fully differentiated and *Ngn2* transcripts are still detected at high levels (thalamic expression of Ngn2 is downregulated postnatally but persists at least until P5; see Supplemental Figure S1 at http://www.neuron.org/cgi/content/full/39/3/439/DC1). Figure 2B shows that the high-rostral to low-caudal gradient of GFP expression persisted at birth in the DT. In order to distinguish between thalamic neurons and neuropil, we have performed double staining of GFP and a neuronal marker (MAP-2; see Figures 2C and 2D). We found that the only nuclei containing Ngn2+ thalamic neurons are rostral thalamic nuclei: (1) the anterior group of thalamic nuclei (AD, AM, AV), (2) the ventro-lateral nucleus (VL), and (3) the ventro-medial nucleus (VM). The common property of these three groups of thalamic nuclei expressing high levels of *Ngn2* is that they all project to rostro-medial cortical areas (Figure 2E; Caviness and Frost, 1980; Crandall and Caviness, 1984; Hohl-Abrahao and Creutzfeldt, 1991): the anterior group (AM-AV-AD) projects to motor area 8 and the rostro-medial cingulate cortex (areas 24, 27, and 29b); the ventro-lateral (VL) nucleus projects to frontal areas 4 and 6; and the ventro-medial nucleus (VMb) projects mainly to frontal cortical areas 31 and 14a.

This analysis shows that *Ngn2* is expressed by a contingent of rostral thalamic neurons that first send their axons to rostral territories of the *ventral telencephalon*

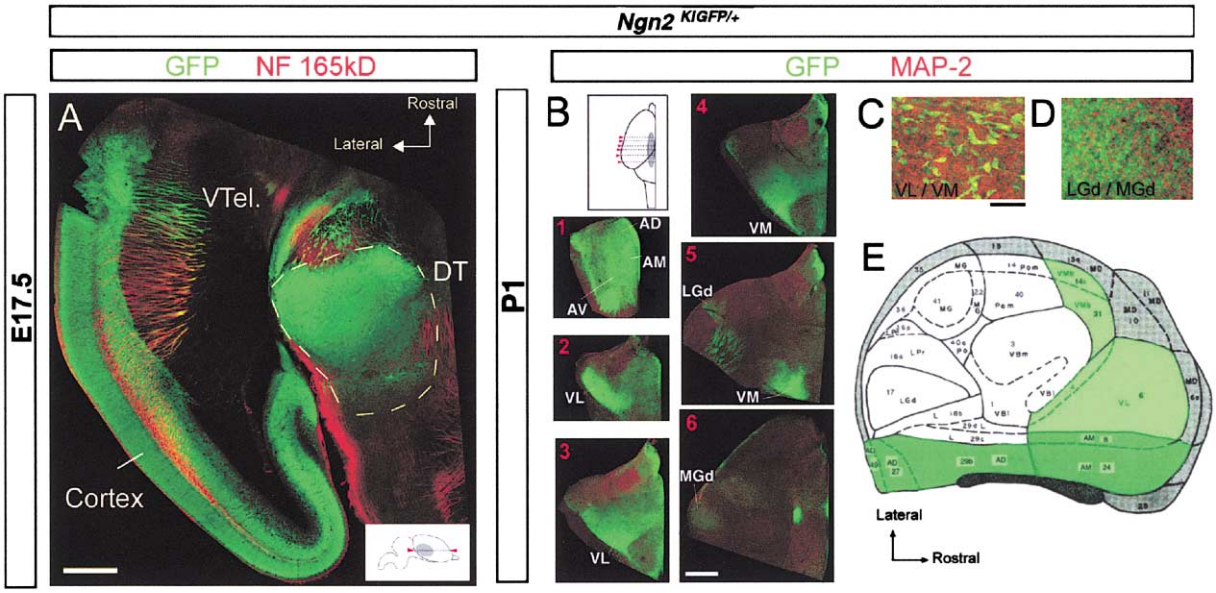

Figure 2. *Neurogenin-2* Is Expressed by Thalamic Neurons Projecting to Rostro-Medial Cortical Areas

(A) Horizontal section through an E17.5 *Ngn2*$^{KIGFP/+}$ embryo illustrating the presence of a high-rostral to low-caudal gradient of *Ngn2* expression in the dorsal thalamus (circled) but not in the cortex. The red counterstaining represents the distribution of axons using anti-Neurofilament 165 kDa staining.

(B) Representative coronal sections taken from rostral (1) to caudal (6) levels of the dorsal thalamus of a postnatal day 1 (P1) *Ngn2*$^{KIGFP/+}$ mouse. At this stage, the transgene is expressed by neurons at high levels in anterior-most thalamic nuclei (AD-AM-AV / VL / VM). Notice that the low level of expression in the more posterior nuclei such as the LGd and the MGd represents neuropil staining and therefore does not represent expression in thalamic neurons (see C and D).

(C and D) Single optical confocal sections illustrating *Ngn2*-expressing neurons in VL and VM thalamic nuclei (double labeling of MAP2^{+} GFP^{+} in C) and the single labeling of GFP^{+} neuropil in thalamic nuclei such as LGd and MGd (negative for MAP-2 in D).

(E) Schema illustrating the pattern of areal projection of thalamic axons emerging from nuclei expressing *Ngn2* (in green). Flatmount representation of cortical areas in the adult mouse modified from Caviness (1975). Numbers identify cortical areas, whereas letters abbreviations indicate the main thalamic nucleus innervating each area.

Scale bars equal 500 μm (A), 400 μm (B), and 25 μm (C and D).

Abbreviations: AM-AV-AD, anteromedial, anteroventral, and anterodorsal thalamic nuclei; LGd, dorsal part of the lateral geniculate nucleus; MGd, medial geniculate nucleus; VL, ventrolateral nucleus; VM, ventromedial nucleus.

and ultimately project to rostral cortical areas and suggested that *Ngn2* could play a role in the specification of neuronal connectivity in the thalamus.

Ngn2 Controls the Topography of Thalamocortical Projections in the Ventral Telencephalon and in the Cortex

The vast majority of *Ngn2* knockout mice die soon after birth, precluding an analysis of the cortical targeting of thalamic axons using classical retrograde axonal tracing in postnatal brains. Therefore, to test directly if *Ngn2* controls the topography of TC axonal projections, we performed anterograde tracing using carbocyanin dye injections restricted to the rostral third of the embryonic DT. A first analysis performed on E17.5 brains sectioned horizontally revealed that the projection of axons from the rostral DT was significantly shifted caudally in the ventral telencephalon of *Ngn2* knockout mice (n = 11; Figure 3B) as compared to control littermates (n = 5; Figure 3A). This analysis also revealed a pronounced pathfinding defect in the ventral telencephalon (VT) of *Ngn2* knockout mice, where axons from the rostral DT navigated rostro-caudally in the IC (arrowheads in Figure 3D), as opposed to the normal medio-lateral projections into the cortex observed in control animals (Figure 3C).

In E16.5 control mice (n = 5), DiI tracing of rostral DT projections resulted in the anterograde labeling of TCAs spanning approximately the rostral half of the VT (black curve in Figure 3E and double arrow in Figure 3G), and consequently the rostral half of the cortex (black curve in Figure 3F and star in Figure 3G). This quantitative analysis revealed that axons emerging from the rostral third of the DT of *Ngn2*$^{-/-}$ embryos (n = 8) were significantly shifted caudally, both in the ventral telencephalon (red curve in Figure 3E and Figures 3I and 3J) and in the cortex (blue curve in Figure 3F and Figures 3I and 3J) as compared to control mice. Therefore, *Ngn2* controls the topography of projection of thalamic axons to rostral territories of the ventral telencephalon and as a consequence to rostral cortical domains. However, *Ngn2* is not only expressed in the rostral territory of the DT but also at the ventro-dorsal telencephalic boundary (VDB) and in the cortex (Fode et al., 2000; see also Figures 1A–1C and 2A). Therefore, at this point it is difficult to conclude if Ngn2 expression is required in the dorsal thalamus to exert its role in the control of TC pathfinding. Later on (Figure 7) we will demonstrate that this caudal shift in the projection of rostral thalamic neurons is largely cell autonomous and therefore that *Ngn2* expression is required in the dorsal thalamus for

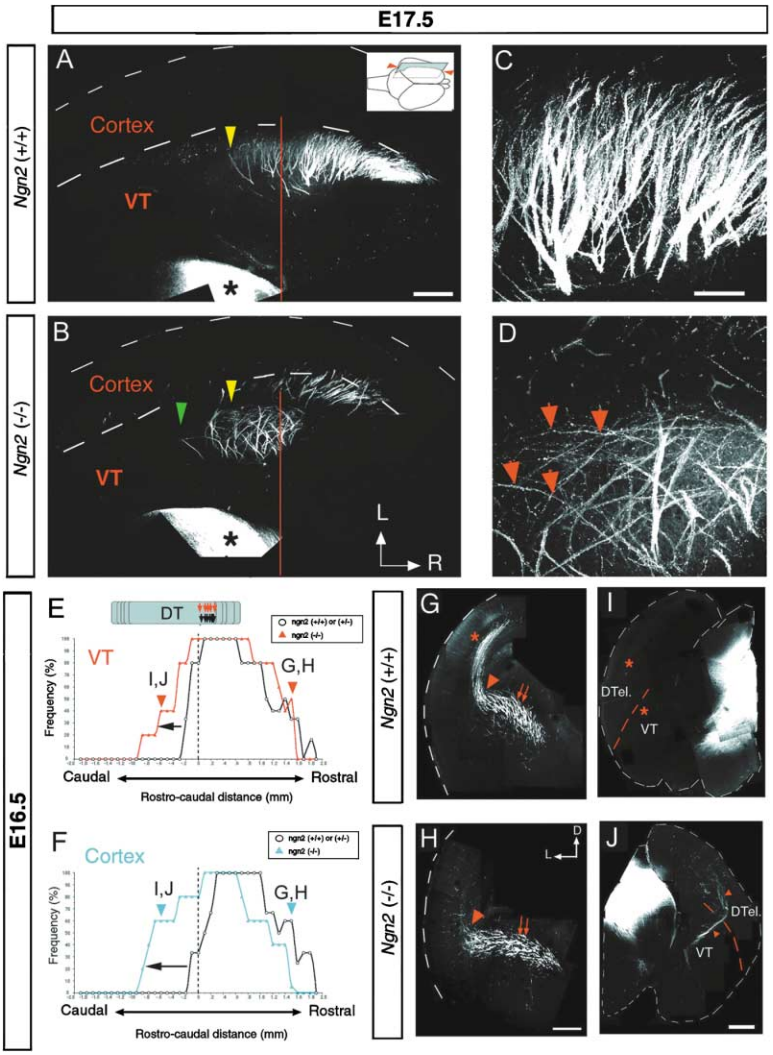

Figure 3. *neurogenin2* Controls the Topography of Thalamocortical Projection In Vivo

(A and B) An oblique section through the internal capsule (indicated in the inset) reveals that the projection of axons originating from rostral DT of *Ngn2*$^{-/-}$ mice (B; n = 11) is shifted caudally in the ventral telencephalon when compared to *Ngn2*$^{+/+}$ mice (A; n = 5). Green and yellow arrowheads indicate the position of the most caudal axons in the VT of Ngn2 knockout and control mice, respectively, relative to a fixed anatomical position (red line: rostral tip of the diencephalon).

(C and D) High-magnification confocal micrograph illustrating that in the internal capsule of *Ngn2*$^{-/-}$ mice, axons are navigating rostrocaudally (arrowheads in D) instead of mediolaterally as observed in control mice (C).

(E and F) Quantification of the rostro-caudal topography of projection of TC axons in the ventral telencephalon (E) and the cortex (F) of coronally sectioned E16.5 control (*Ngn2*$^{+/+}$; n = 5) or mutant embryos (*Ngn2*$^{-/-}$; n = 8). The reference point (0 mm) is indicated by a vertical dotted line and corresponds to the first section where TC axons are traveling from the diencephalon to the ventral telencephalon. The gray boxes shown above the graph in (E) represent the rostro-caudal extent of the DT in all cases examined as well as the rostro-caudal position of the injection sites for *Ngn2*$^{-/-}$ mice (red arrows) and control mice (black arrows). On the *y* axis, data are expressed as the frequency of sections (expressed as a proportion of all animals examined) where fibers are found in the ventral telencephalon (E) or in the cortex (F).

(G–J) Confocal projections at a rostral level (G and H; 1.5 mm rostral to reference point; see arrowheads in E and F) and at a caudal level (I and J; 0.6 mm caudal to the reference point, indicated by the arrowheads in E and F) of coronal sections of control (G and I) and *Ngn2*$^{-/-}$ (H and J) mice illustrating the caudalization of TC projections originating from the rostral thalamus of *Ngn2*$^{-/-}$ (G) compared to wild-type (H) mice. The arrowheads in (J) point to thalamocortical axons in the ventral telencephalon and in the cortex never found at the corresponding level in the control mice (asterisks in I).

Scale bars equal 300 μm (A and B), 100 μm (C and D), and 200 μm (G–J).

the specification of the topography of thalamic neurons projections in the ventral telencephalon.

Neurogenin2 Specifies the Ability of Rostral Thalamic Neurons to Enter into the Cortex

In E16.5 control mice, axons originating from rostral territories of the DT had reached rostral levels of the telencephalon by navigating rostrally in the IC (double arrows in Figure 3G) and had already crossed the VDB of the telencephalon (arrowhead in Figure 3G) to enter the cortex (star in Figure 3G). Strikingly, in E16 *Ngn2* knockout mice, TCAs had reached the same rostral level of the VT to form the IC (double arrows in Figure 3H), but they did not cross the VDB (arrowhead in Figure 3H) and were thus unable to enter the cortex at this rostral level. This pathfinding defect was very specific for axons originating from rostral DT, as axons originating from caudal DT were able to cross the VDB at the same level of section (see Supplemental Figures S2A–S2C at http://www.neuron.org/cgi/content/full/39/3/439/DC1). This result indicated that *Ngn2* controls the response of axons from rostral (but not caudal) DT to the extracellular cues encountered at the VDB of the telencephalon, a response that normally enables DT axons to invade the cortex. Because *Ngn2* is expressed both by neurons in rostral territories of the DT and at the VDB of the telencephalon in the environment of the TCA (Fode et al., 2000; see also Figure 2A), the pathfinding defect affecting TC axons could be cell autonomous or cell nonautonomous. However, the fact that only axons from rostral but not caudal DT were stalled at the VDB suggested that the defect is mainly cell autonomous and that extracellular signals from the boundary were intact in *Ngn2* knockout mice. Later on we demonstrate directly that *Ngn2* expression is required in thalamic neurons in order to specify their ability to enter the cortex (see Figure 7).

In *Ngn2* knockout mice, axons from rostral DT also showed a pronounced defasciculation defect in the IC (see Supplemental Figures S2D–S2F). Again, at the same

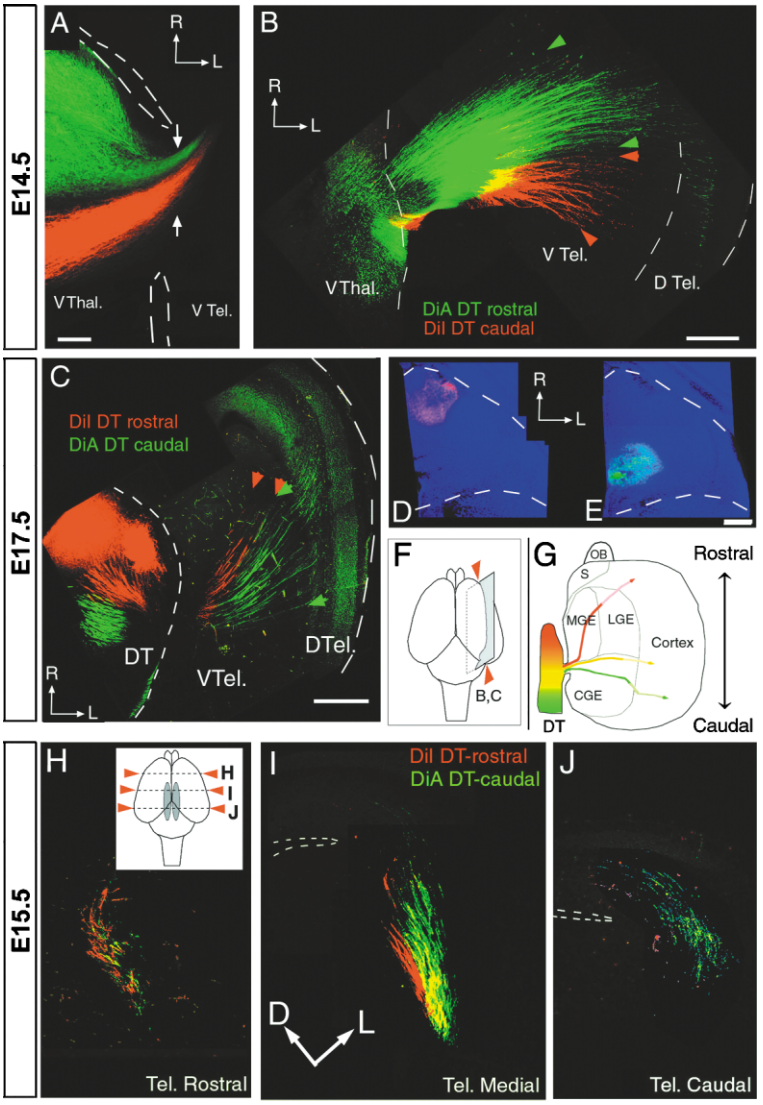

Figure 4. The Tangential Specificity of Thalamocortical Projections Is Initiated in the Ganglionic Eminence

(A) Thalamocortical axons (TCA) leave the diencephalon to enter the telencephalon through a narrow passage of about 300 μm wide in the rostro-caudal axis (between the two arrows). Horizontal section of an E15 embryo injected with DiA in the rostral part of the dorsal thalamus (DT) and DiI in the caudal part.

(B) At E15, TCA emerging from the rostral part of DT (green, DiA) are segregated from the TCA emerging from the caudal part of the DT (red, DiI) in the ganglionic eminence (VT). Oblique sections passing through the plan formed by the internal capsule as depicted in (F).

(C–E) The rostro-caudal segregation of TCA emerging from different part of the DT along the rostro-caudal axis is maintained at later stages (E17.5 embryo sectioned obliquely as shown in F). The size and position of representative injection sites in the DT are illustrated in (D) (DiI) and (E) (DiA) of an E17.5 embryo (blue, bis-benzimide counterstaining).

(F) Schematic dorsal view of an embryonic mouse brain illustrating the oblique plan of section used in (B) and (C).

(G) Schematic view of a "flattened" telencephalic vesicle illustrating the rostro-caudal segregation of axons originating from different rostro-caudal levels of the DT (see text).

(H–J) TCA originating from distinct rostro-caudal territories are also segregated along the dorsoventral axis. In the ventral telencephalon, axons originating from rostral territories of the DT (DiI, red in H and I) are found at more rostral levels of sections (H and I) than axons emerging from caudal DT (DiA, green I and J). At intermediate levels of section (I) where the two contingents overlap, axons from rostral DT (red) are systematically located more dorsally than axons emerging from the caudal DT (green).

Abbreviations: MGE, medial ganglionic eminence; LGE, lateral ganglionic eminence; CGE, caudal ganglionic eminence; VThal., ventral thalamus; VTel., ventral telencephalon; DT, dorsal thalamus; S, septum; OB, olfactory bulb; Tel., telencephalon.

Scale bars equal 80 μm (A), 250 μm (B, H, and I), 700 μm (C), 250 μm (D and E).

level, axons from caudal DT did not show any defect of fasciculation, suggesting that *Ngn2* specifically determines the responsiveness of axons from the rostral and not caudal thalamus.

Interestingly, the dorsoventral segregation of axons emerging from rostral and caudal territories of the DT was not affected in *Ngn2* knockout mice: axons originating from rostral DT were always located dorsal to the axons originating from caudal DT (see white arrows in internal capsule, Supplemental Figures S2A–S2C at http://www.neuron.org/cgi/content/full/39/3/439/DC1) as in control mice (compare with Figure 4I). This result shows that the segregation of TC axons in the rostro-caudal and the dorsoventral plans are not controlled by the same molecular mechanisms.

Role of the Ventral Telencephalon in the Initiation of the Topography of Thalamocortical Projection

The results shown above suggested that rostral thalamic axons project to rostral cortical territories under the influence of topographic cues present at the level of an intermediate target, the VT. We first reexamined carefully how the topography of TC projections emerged during early stages of development. To do this, we performed restricted double injections of carbocyanin lipophilic dyes (DiI-red and DiA-green) at different rostro-caudal levels of the developing DT. Note that the injections were carefully performed along a strict latero-medial axis (see Figures 4D and 4E) using a saggital approach. In mice, the first TCAs emerge from the DT around E13.5, pioneer the VT around E14, and reach the boundary between the ventral and the dorsal part of the telencephalon (VDB) between E14.5 and E15.5 in the mouse (J.S. and F.P., unpublished data; Bicknese et al., 1994; Molnar et al., 1998b; Molnar and Blakemore, 1995b). Axons emerging from rostral or caudal territories of the developing DT navigate from the diencephalon to the VT through a narrow passage of about 300 microns at E14–E15 (Figure 4A). Surprisingly, we found that this redistribution is highly organized along the rostro-

caudal axis: TC axons from rostral DT were directed rostrally when entering the VT, whereas axons from caudal DT project more caudally in the VT at E14.5 (Figure 1B). This level of rostro-caudal segregation is not transient during development, and at later stages (E17.5), when most TCAs have reached their final cortical targets, it is still observed in the VT (see Figure 4C).

TCA emerging from rostral thalamic territories were also segregated from the axons emerging from caudal thalamus along the dorsoventral axis in the VT. Figures 4H–4J show that at a rostral level of the GE, only TC axons from rostral DT were found (Figure 1H), whereas at an intermediate rostro-caudal level (Figure 4I), TC axons originating both from rostral and caudal parts of the DT were present but were segregated along the dorsoventral axis: TC axons from rostral DT were always more dorsal then TC axons from caudal DT. Finally, at more caudal levels of the ganglionic eminence in the VT (Figure 4J), only axons from the caudal DT were found.

A New In Vitro Assay to Study the Development of the Topography of TC Projections

Our results suggest that the topography of TC axon projections to different cortical domains is established prior to their arrival in the cortex, possibly under the influence of ventral telencephalon-derived guidance cues (Figure 4G). To test this possibility more directly, we developed a new "telencephalic whole-mount" assay. Briefly, this assay consists of coculturing thalamic explants from 250 μm thick vibratome slices (DT1 to DT5, see Supplemental Figure S3 in accompanying article by Dufour et al., 2003 [this issue of *Neuron*]) isolated from GFP-expressing mice with whole-mount telencephalic vesicles isolated from wild-type mice (see schema in Figure 5D and Experimental Procedures). This assay enables the two-dimensional visualization of TC axons pathfinding in the telencephalon in vitro.

Our results show that axons emerging from the most rostral regions of E14.5 DT (DT1 and DT2 pooled as DTR) grew rostrally in the ventral telencephalon (Figure 5A). Thalamic axons emerging from progressively more caudal levels of the DT showed a less pronounced tendency to grow rostrally in the ventral telencephalon (Figure 5B). In contrast, axons originating from the most caudal levels of the DT (DT5, also called DTC) exhibited the opposite trend, growing preferentially in the caudal part of the ventral telencephalon to invade subsequently more caudal cortical territories (Figure 5C). The topography of TC projections was quantified by measuring the distribution of the mean normalized optical density emerging from the GFP signal (normalized OD; see Experimental Procedures) along the rostro-caudal axis of the ventral telencephalon (normalized distance, 40 bins; Figure 5D). Figures 5E and 5F demonstrate that the spatial distribution of axons emerging from DTM and DTC is significantly shifted caudally in the VT compared to DTR.

These results demonstrate that the topography of TC projections is initiated by extracellular cues located in the ventral telencephalon, the main intermediate target of TC axons. For rostral thalamic axons, these ventral telencephalon-derived cues could be a combination of chemo-attractive cues located in the rostral part of the ventral telencephalon and/or chemo-repulsive cues located in the caudal part of the ventral telencephalon.

Neurogenin-2 Specifies the Topography of Thalamocortical Projections Cell Autonomously

As shown above, *Ngn2* is not only expressed in the rostral DT but also at the ventrodorsal telencephalic boundary and in the cortex (see Figures 1A–1C and Chapouton et al., 2001; Fode et al., 2000; Nieto et al., 2001); therefore, it is difficult to conclude if it exerts its role in TC pathfinding cell autonomously or non-cell autonomously. We therefore used the telencephalic whole-mount assay to test the cell autonomy of *Ngn2* action in TC pathfinding. We cocultured E14.5 wild-type telencephalon with isochronic rostral slices of dorsal thalami (DTR) isolated from either wild-type mice expressing GFP under the control of a ubiquitous β-actin promoter (Okabe et al., 1997) or $Ngn2^{KIGFP/KIGFP}$ mutant mice. We could not use $Ngn2^{KIGFP/+}$ rostral thalamic explants because the level of fluorescence after 3 days in vitro was too low for proper quantification (data not shown).

As shown above (Figure 5), after 3 days in vitro, axons from rostral DT of wild-type mice were reproducibly growing rostrally in the ventral telencephalon, mimicking the in vivo situation (Figures 6A and 6B). In contrast, axons originating from rostral DT of $Ngn2^{KIGFP/KIGFP}$ embryos display striking pathfinding errors when growing in a wild-type telencephalic environment (Figures 6C and 6D). Some axon fascicles are growing caudally (arrowhead in Figure 6C) or medially (arrowhead in Figure 6D) in the ventral telencephalon, ultimately invading more caudal cortical domains. The quantification demonstrated that the topography of projection of $Ngn2^{KIGFP/KIGFP}$ axons from rostral DT growing in a wild-type ventral telencephalon was significantly shifted caudally compared to $Ngn2^{+/+}$ axons (Figure 6E). This demonstrates that *Ngn2* specifies the connectivity of thalamic neurons by controlling the responsiveness of their axons to topographic cues encountered in the ventral telencephalon.

Examination at higher magnification showed that some of the $Ngn2^{KIGFP/KIGFP}$ axons were repelled by the wild-type VDB and were growing in the rostro-caudal plan instead of reaching more dorsal cortical territories (Figures 6F and 6G), demonstrating that the conversion of the response of thalamic axons at this boundary observed in vivo is also largely cell autonomous.

Loss of *Neurogenin2* Expression Does Not Respecify the Molecular Identity of Dorsal Thalamic Neurons

We wanted to evaluate if the change of thalamic neurons connectivity due to the loss of *Ngn2* could be a secondary consequence of a more general respecification of neuronal identity in the rostral thalamus. To test this, we documented the pattern of expression of four transcription factors (*Gbx2*, *Lhx2*, *Dlx1*, *Id2*) previously involved in thalamic regionalization at two different stages of neuronal development: E12.5–13.5 when the majority of thalamic precursors are still proliferating and at E15.5 when the majority of thalamic neurons are postmitotic (Angevine, 1970). Briefly, *Gbx2* is a homeodomain-containing transcription factor involved in the specification of thalamic neuronal connectivity (Hevner et al., 2002; Miyashita-Lin et al., 1999; Nakagawa and O'Leary, 2001);

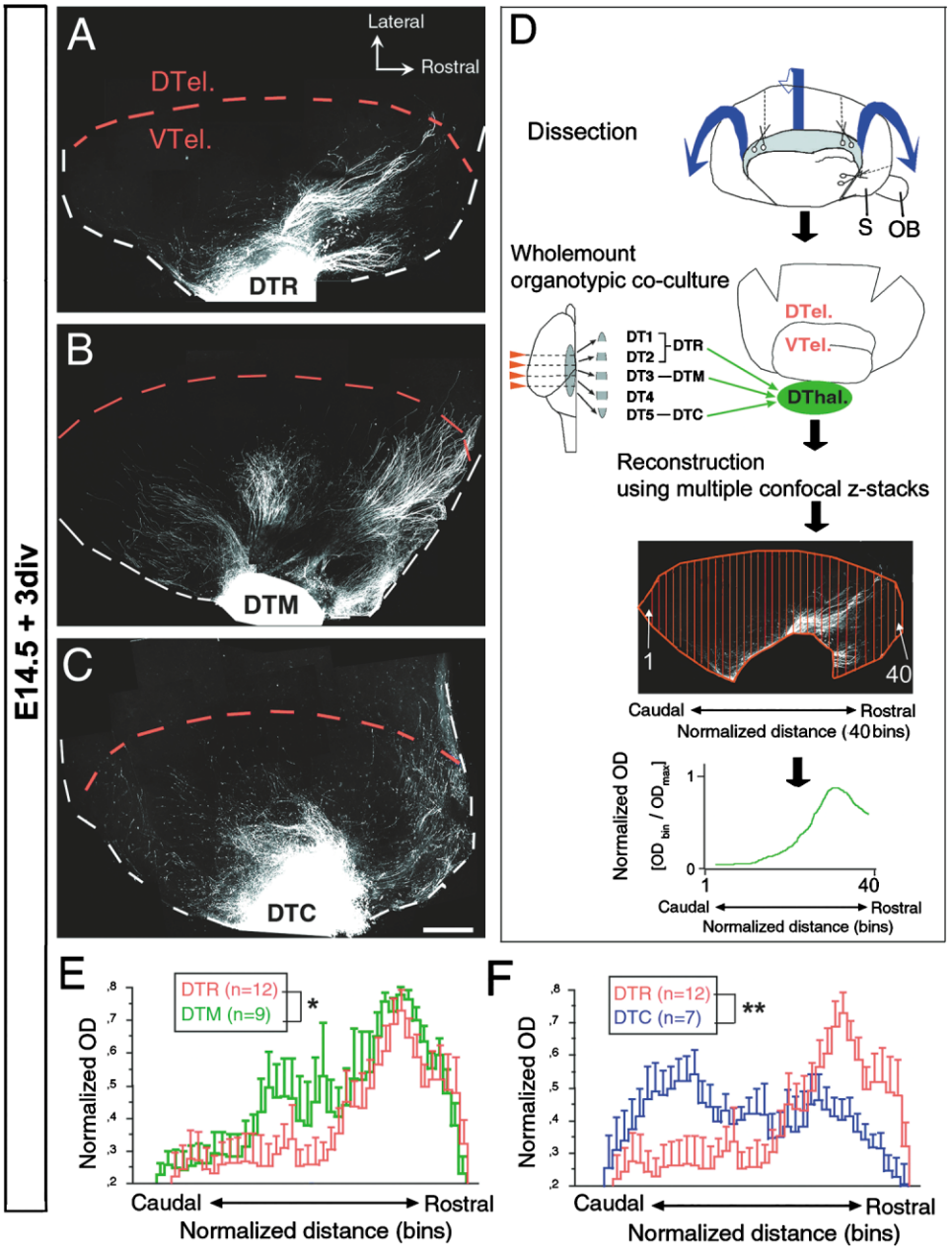

Figure 5. The Telencephalic Whole-Mount Assay Recapitulates the Topography of Thalamocortical Axons Projection In Vitro

(A–C) Following the experimental paradigm depicted in (D), we developed an assay where axons emerging from rostral slices of E14.5 DT (DTR in A) or progressively more caudal parts (DTM in B and DTC in C) of GFP-expressing DT are cocultured for 3 days in vitro (3 div) with a telencephalic whole-mount. This reveals that different rostro-caudal levels of the DT are responding differently to intermediate cues present in the VT.

(D) Schema illustrating the experimental paradigm. To quantitate the behavior of GFP^{+} thalamic axons in different experimental conditions, we performed normalized optical density measurement of the fluorescent GFP signal in 40 bins of equal width distributed along the rostro-caudal axis of the ventral telencephalon (normalized distance; see Experimental Procedure for details). Each montage is composed of 20–30 confocal z-stacks adjacent images (maximum projection) of nonsaturating 8-bit gray levels.

(E and F) Quantitative analysis of the topography of thalamocortical axon outgrowth in the ventral telencephalon using normalized GFP-derived optical density measurement (*y* axis) performed in 40 bins of normalized width (*x* axis) distributed along the rostro-caudal axis of the ventral telencephalon for axons originating from different rostro-caudal levels of the DT (red, DTR explants; green, DTM explants; and blue, DTC explants). This demonstrates that the rostral third of the DT (DTR) grows significantly more rostrally than progressively more caudal parts of the DT (two-way ANOVA test: DTR versus DTM *$p < 0.02$; DTR versus DTC **$p < 0.001$). Error bars indicate standard error to the mean and *n* represents the number of explants reconstructed for the optical density measurements from at least three independent experiments.

Abbreviations: DTel, dorsal telencephalon; VTel, ventral telencephalon; DThal, dorsal thalamus; S, septum; OB, olfactory bulb.

Scale bar equals 500 μm (A–C).

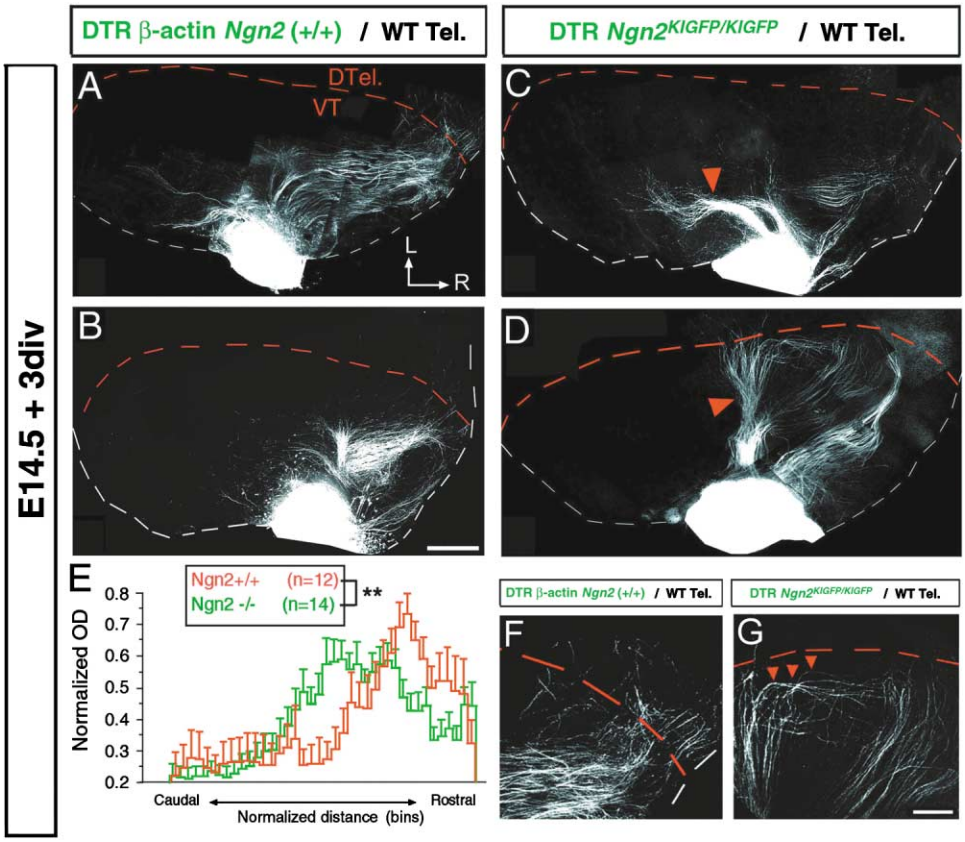

Figure 6. *Neurogenin2* Specifies the Topography of Thalamocortical Projection Cell Autonomously

(A–D) Two representative examples of the topography of projection of E14.5 $Ngn2^{+/+}$ (A and B) and $Ngn2^{-/-}$ (C and D) thalamocortical axons from rostral thalamic slices (DTR) into wild-type telencephalic whole-mount explants. This shows that after 3 div, axons from rostral territories of the DT have a strong bias to grow rostrally in the ventral telencephalon, whereas rostral thalamic neurons defective for *Ngn2* tend to project more caudally in the ventral telencephalon (arrowheads in C and D).

(E) Quantitative analysis of the topography of axon projection of $Ngn2^{+/+}$ (n = 12 explants from three independent experiments) and $Ngn2^{KIGFP/KIGFP}$ (i.e., $Ngn2^{-/-}$; n = 14 explants from three independent experiments) rostral thalamic (DTR) explants in wild-type ventral telencephalon. Each curve represents the average of the normalized optical density measures from 40 bins distributed along the rostro-caudal axis of the ventral telencephalon for GFP-expressing axons originating from wild-type $Ngn2^{+/+}$ GFP-expressing rostral thalamus (DTR; red curve) and $Ngn2^{KIGFP/KIGFP}$ (i.e., $Ngn2^{-/-}$) axons (control versus knockout, ANOVA **$p < 0.001$).

(F and G) High-power view illustrating the behavior of $Ngn2^{+/+}$ (F) and $Ngn2^{-/-}$ thalamic axons at the ventrodorsal boundary of the telencephalon. The majority of $Ngn2^{-/-}$ thalamic axons are repelled by the wild-type boundary, showing that *Ngn2* specifies also cell autonomously the ability of thalamic axons to enter into the cortex.

Scale bars equal 700 μm (A–D) and 100 μm (F and G).

Lhx2 is a LIM-containing transcription factor previously involved in the regionalization of the dorsal thalamus (Nakagawa and O'Leary, 2001); *Dlx1* is a homeodomain protein and marker of GABAergic interneurons normally expressed in the ventral and not dorsal thalamus (Bulfone et al., 1993; Garel et al., 2002; Warren and Price, 1997); and *Id2* is a member of the HLH transcription factor family previously implicated in early DT regionalization (Miyashita-Lin et al., 1999). The expression of these four transcription factors was unchanged in *Ngn2* knockout mice both in thalamic precursors (E12.5–E13.5; Figures 7A–7H) and postmitotic thalamic neurons (E15.5; Figures 7I–7L). This strongly suggests that in the absence of *Ngn2* dorsal thalamic precursors and neurons are not respecified at the molecular level and therefore that in the dorsal thalamus *Ngn2* specifies neuronal connectivity in a rather specific manner unlike in the cortex where both its *proneural* and cell fate specification functions are predominant (Fode et al. 2000; C.S., O. Armant, O. Britz, J.S., F.P., and F.G., unpublished data).

Discussion

Role of bHLH Transcription Factors in the Specification of Neuronal Subtype Identity

Studies of motor neuron development in the vertebrate spinal cord have begun to elucidate the genetic mechanisms involved in the specification of motor neuron projections. Specific LIM homeodomain (LIM-HD) transcription factors play an instructive role in establishing motor neuron subtype identity (Jessell, 2000; Shirasaki and Pfaff, 2002). Importantly, it has recently been shown that the combinatorial expression of LIM-HD transcription factors confers motor neuron subtypes with the ability to select specific axon pathways to reach their distinct

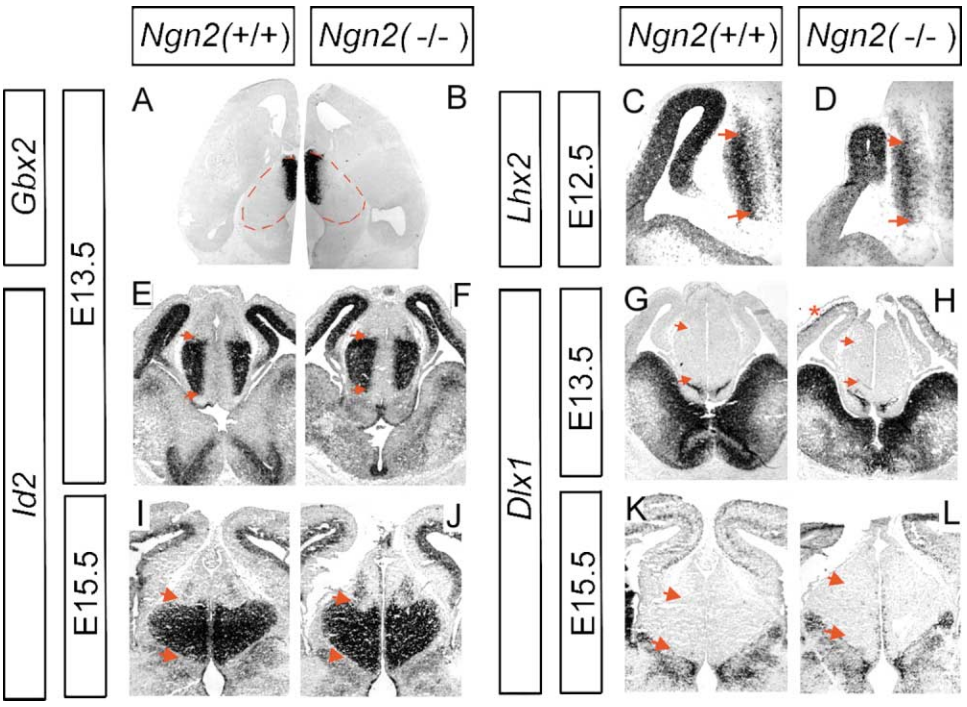

Figure 7. *Neurogenin2* Loss of Function Does Not Respecify the Molecular Identity of Thalamic Neurons

In situ RNA hybridization for *Gbx2* (horizontal sections in A and B at E13.5), *Lhx2* (coronal sections in C and D at E12.5), *Id2* (coronal sections; E and F at E13.5 and I and J at E15.5), and *Dlx1* (coronal sections; G and H at E13.5 and K and L at E15.5). Note that mRNA distribution is unchanged between control (panels A, C, E, G, I, and K) and *Ngn2* knockout (panels B, D, F, H, J, and L) mice for these four transcription factors at E12.5–E13.5 when thalamic progenitors are still proliferating and E15.5 when most thalamic neurons are postmitotic (Angevine, 1970). The circled area in (A) and (B) indicates the extent of the dorsal thalamus. Arrows indicate the dorsoventral extent of the dorsal thalamus, and the asterisk in (H) indicates the upregulation of *Dlx1* mRNA level in the cortex previously reported elsewhere (Fode et al., 2000).

muscle targets (Kania et al., 2000; Sharma et al., 2000; Thaler et al., 2002). Another study showed recently that the murine POU domain transcription factor Brn-3.2 regulates pathfinding by retinal ganglion cell (RGC) axons at multiple points along their pathway, as well as the final establishment of topographic order in their target, the superior colliculus (Erkman et al., 2000). Interestingly, in the developing spinal cord and in the telencephalon, bHLH transcription factors have been primarily shown to control cell cycle exit and to mediate neuronal versus glial cell fate choices (reviewed in Bertrand et al., 2002). However, recently the bHLH transcription factors *Ngn2* and *Olig-2* have been shown to cooperate in the specification of motorneuron phenotypes (Novitch et al., 2001; Zhou and Anderson, 2002). Again in these studies, *Ngn2* was shown to act mainly at the level of motorneuron progenitors, activating panneuronal traits, but in light of our results it would be interesting to determine if *Ngn2* also plays a role in the specification of motorneuron connectivity.

To date, the main limitation for studying the roles of *proneural* genes in axon pathfinding has been that in most models, defects in neurogenesis preclude the analysis of neuronal connectivity. This is not the case in the DT of *Ngn2* mutants, possibly because the normal *proneural* function of *Ngn2* is compensated for by *Ngn1,* which is constitutively expressed in the DT, and/or by *Mash1,* which is upregulated in early DT progenitors in *Ngn2* mutants at E12–E13 and is responsible for the misspecification of a small subset of DT neurons toward a GABAergic phenotype (Fode et al., 2000). The present study demonstrates that *Ngn2* also has other functions, controlling thalamic neuronal connectivity, possibly through the transcriptional control of the expression of receptors or intracellular signaling molecules involved in axon guidance. Previous investigations have shown that several important neuronal traits such as cell body position or connectivity are specified in dividing precursors (McConnell, 1988; McConnell and Kaznowski, 1991; Shirasaki and Pfaff, 2002). Future investigations will be focused at exploring the temporal requirement of *Ngn2* expression in the specification of neuronal connectivity: based on its pattern of expression, *Ngn2* expression could be required in thalamic progenitors and/or postmitotic neurons.

Our results demonstrate that *Ngn2* specifies the pattern of thalamic neuronal projection in a cell-autonomous manner, by controlling axon responsiveness to intermediate cues located in the ventral telencephalon. This represents the first evidence for such a role in mammals but could actually represent a phylogenetically conserved mechanism. In *Drosophila, atonal,* a close ortholog of mammalian *Ngns* (reviewed in Bertrand et al., 2002) acts as a proneural gene in the chordotonal organs in the peripheral nervous system (PNS) and the larval and adult photoreceptor organs (Chien et al., 1996; Jarman et al., 1993, 1994). However, Hassan et al. (2000) have shown that in contrast to its function in the PNS,

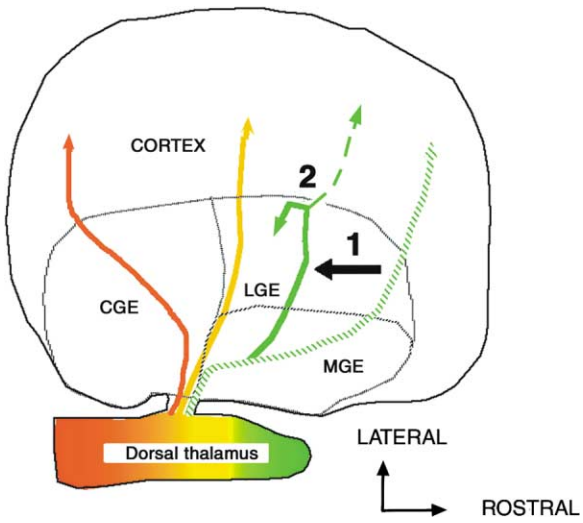

Figure 8. Proposed Model of Specification of the Topography of Thalamocortical Projections

Axons originating from distinct rostro-medial to caudo-lateral territories of the dorsal thalamus (pseudo-colored arbitrarily from green to orange) are sorted to distinct cortical domains by topographic cues located in an intermediate target, the ventral telencephalon (MGE, LGE, and CGE). Inactivation of *Ngn2* in the rostral domain of the thalamus has two main consequences on TCA pathfinding: (1) a caudalization of rostral DT projections in the ventral telencephalon, and (2) the arrest of the majority of rostral thalamic axons at the ventrodorsal boundary of the telencephalon, preventing them from entering the cortex.

atonal does not act as a proneural gene in the embryonic brain but instead regulates axon arborization and pathfinding during larval and pupal development by interacting with *Notch* (Giniger, 1998; Giniger et al., 1993; Hassan et al., 2000). A recent study has further shown that *atonal* controls axon branching by regulating the level of expression of *Robo3* a receptor for *Slit*. This again strongly argues for a dual function of bHLH transcription factors (1) in the specification of panneuronal properties and cell cycle exit (reviewed in Anderson, 1999; Bertrand et al., 2002) and (2) in the specification of unique neuronal traits, including connectivity.

Role of the Ventral Telencephalon in the Patterning of the Topography of Thalamocortical Projections

Our results provide the first direct demonstration that the ventral telencephalon contains topographic cues playing a central role in the patterning of thalamic axon projections to specific cortical domains (see Figure 8). Several previous lines of evidence supported a general role for the ventral telencephalon in TCA pathfinding. First, in vitro, the VT behaves as an intermediate target exhibiting a chemotropic effect on the outgrowth of thalamic as well as cortical axons (Braisted et al., 1999; Metin and Godement, 1996; Richards et al., 1997), a chemotropic action thought to be mediated by Netrin-1 (Braisted et al., 2000; Metin et al., 1997). Second, pioneer axons emerging from early ventral telencephalic neurons invade both the cortex and the thalamus and could play a role in the reciprocal guidance of TC and CT axons (Metin and Godement, 1996; Molnar and Cordery, 1999). Third, recent results have shown that knockout mice for transcription factors involved in the regionalization of the ventral telencephalon such as *Ebf1, Dlx1/2*, and *Mash1* are characterized by defective TC pathfinding. In these knockout mice, TCAs are stalled at the interface between the ventral thalamus and the ventral telencephalon (*Mash-1*; Tuttle et al., 1999) or in the ventral telencephalon proper (*Ebf-1*; Garel et al., 1999; *Dlx1/2*; Garel et al., 2002). These phenotypes are most likely cell nonautonomous and are due to the aberrant patterning of gene expression in the ventral telencephalon characterizing these mutant mice (Casarosa et al., 1999; Garel et al., 1999, 2002; Marin et al., 2002; Tuttle et al., 1999).

Our results demonstrate that intermediate target-derived cues can control the topography of axonal projections. This contrasts with the retino-tectal system where the cues that dictate the antero-posterior topography of retinal projections are contained exclusively in their final target, the tectum (Flanagan and Vanderhaeghen, 1998), but has some interesting parallels with the innervation of the limb where a region proximal to the developing limb, the plexus, has been shown to initiate the topography of muscle innervation by motoneuron axons (Kania et al., 2000; Landmesser, 2001).

Presorting of Thalamocortical Axons in the Ventral Telencephalon versus Cortical Area-Specific Cues?

About a decade ago, Molnar and Blakemore (1991) have shown that when E16 presumptive thalamic nuclei such as dLGN is cocultured directly with a target (visual) or nontarget (motor) postnatal cortical slices, thalamic axons invade nonspecifically both cortical explants. These authors concluded that cortical areas do not contain area-specific cues sufficient for thalamic axons to differentiate between target and nontarget tissue. However, there are at least three other alternative, nonexclusive interpretations to these results: (1) cortical areas do express these tags but only prenatally when thalamic axons first contact the subplate (around E18 in rat) and not anymore at the time these authors isolated the cortex (P5–P10); (2) such area-specific cues are indeed expressed by cortical areas postnatally but thalamic axons are unresponsive to these cues unless they have been exposed to other intermediate cues (this inductive model of axon responsiveness by intermediate cues has been demonstrated for commissural axons in the spinal cord; Zou et al., 2000); or (3) the inter-areal topography of thalamic projection is initiated by intermediate extracortical cues absent when the thalamic nuclei are cocultured directly with isolated cortical explants as performed by Molnar and Blakemore (1991).

Our experiments directly test this third interpretation and clearly demonstrate that thalamic axons are predirected to distinct cortical domains (motor versus visual, for example) in response to intermediate cues present in the ventral telecephalon, which was removed from the in vitro cocultures performed by Molnar and Blakemore (1991). Future investigations will be aimed at testing if the ventral telencephalon also induces a change in the responsiveness to putative area-specific cues using the same in vitro assay, which would explain the lack of

specificity of thalamocortical projection in the absence of this intermediate target.

In a parallel study (Dufour et al., 2003 [this issue of *Neuron*]), we demonstrate that graded expression of ephrins in the VT act as topographically specific repellents for early TCAs that display graded levels of EphA receptors. Future experiments will test if *Ngn2* exerts its role in the specification of TC connectivity by controlling directly or indirectly Eph receptor expression or signaling, or if *Ngn2* acts upstream of an Eph-independent transcriptional cascade.

Experimental Procedures

Animals

The morning of vaginal plug detection is considered as the morning of the first day of gestation (E0.5). Heterozygous transgenic males expressing EGFP under the control of a ubiquitous chicken β-actin promoter and a CMV enhancer (Okabe et al., 1997) were crossed with OF1 wild-type females (Iffa-Credo, France) in order to obtain litters containing on average 50% GFP^- embryos and 50% GFP^+ embryos. We also bred transgenic mice where EGFP ($Ngn2^{KIGFP}$) or β-galactosidase ($Ngn2^{KIlacZ}$; described in Fode et al., 2000) have been knocked into the endogenous *Ngn2* locus by homologous recombination in ES cells. In the present article the $Ngn2^{KILacZ}$ allele is referred to as *Ngn2*.

Organotypic Coculture

In order to visualize the topography of TC projections in vitro, we developed a coculture assay where alternate 250 μm thick vibratome slices of DT were isolated from mice ubiquitously expressing GFP (Okabe et al., 1997) or $Ngn2^{KIGFP/KIGFP}$ mice (see Supplemental Figure S3 in Dufour et al., 2003 [this issue of *Neuron*]). Slices were then placed adjacent to whole-mount telencephalic vesicles of the same age (isochronic coculture; see Figure 5D). Wild-type embryos were microdissected in cold HBSS; the diencephalon, septum, and olfactory bulb were removed; and the whole telencephalic vesicle was placed on cell culture inserts (1 μm pore size-PET membranes; Becton Dickinson). Organotypic cocultures were performed using an air-interface protocol detailed elsewhere (Polleux and Ghosh, 2002) and were maintained in a 5% CO_2-humidified incubator for at least 3 days in vitro (div).

Immunofluorescence and Confocal Microscopy

After 4% PFA fixation overnight, double labeling was performed on cocultures or 100 μm slices obtained from $Ngn2^{KIGFP}$ mice using a protocol described in detail elsewhere (Polleux and Ghosh, 2002). Briefly, permeabilization was performed by overnight incubation in a blocking solution (Triton Buffered Saline [TBS] 0.05 M where 0.3% Triton X-100 and 3% bovine serum albumin [Sigma] were added). The main antibodies used in this study were polyclonal rabbit anti-GFP (1:2000, polyclonal, Molecular Probes) and monoclonal mouse anti-neurofilament 165 kDaA (1:300; clone 2H3; Developmental Studies Hybridoma Bank). Primary antibodies were visualized using Cy3- (red) or Cy2 (green)-conjugated secondary antibodies (goat anti-mouse or goat anti-rabbit; 1:400, Jackson ImmunoResearch). Sections where observed with a LEICA TC SP2 confocal microscope equipped with helium-neon and argon lasers.

Normalized Optical Density Measurement of GFP Immunofluorescence

In order to alleviate inter-explant variations of size and GFP fluorescence levels, we have first normalized the rostro-caudal extent of the ventral telencephalon by dividing it into 40 vertical oriented bins of equal width (sampling the entire latero-medial extent of the ventral telencephalon; see schema Figure 5D). Second, we have normalized the optical density (OD) measures of the GFP immunofluorescent signal using 8-bit encoded grayscale nonsaturated confocal z-stacks montages using NIH image software. In order to alleviate interexperiment variability of the absolute level of fluorescence, individual OD measures in each 40 bins were normalized to the maximal OD value (OD_{Max}) on each coculture ($OD_{norm} = OD_{bin}/OD_{max}$). The error bars represent standard error to the mean for each OD_{norm} values obtained in each bin of 7 to 14 explants from a minimum of three independent experiments.

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